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Isolation of Intragenic Suppressor Mutations of a Dominant-Negative *ftsZ* Allele

by

Carla Ciccone

**A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science**

**Duquesne University
2003**

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Degree: Master of Science

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ABSTRACT

Streptomyces coelicolor, a gram-positive bacterium containing division genes that are dispensable for growth and viability. *ftsZ* encodes the earliest acting cell division protein in prokaryotes. In *S. coelicolor* a mutation at codon 275 in *ftsZ* (*ftsZ25*), changes an alanine to a valine causing a block in division. Using a homology model of the crystal structure of FtsZ, I hypothesized that changing another amino acid having direct interaction with amino acid 275 might restore function. The structure-directed mutation changed a leucine to a valine at codon 259. The double mutation did not restore division to the organism; this change alone also affected cell division in a negative way. Alternatively, I used two random mutagenesis approaches. One, a chemical mutagen, hydroxylamine yielded no significant results in preliminary experiments. The second way was through the use of a mutator strain form which I isolated two suppressors of *ftsZ25* that partially restore division.

INTRODUCTION

Cell division is an essential process in prokaryotic organisms, such as *Escherichia coli*. For most prokaryotes, cell division occurs by binary fission. In *E. coli*, mutations in cell division genes have a detrimental effect on the growth and viability of the organism. However, cell division genes in *Streptomyces coelicolor* are not required for growth and viability of the organism. For this reason, *S. coelicolor* is a useful model organism to study cell division and the genes which are involved.

My thesis research involved isolation of suppressor mutations of a dominant-negative mutation in *ftsZ*, called *ftsZ25*, which will be referred to as *ftsZ*(A275V). The mutation, which changes an alanine to a valine at position 275 of the 399 amino acid protein, causes the organism to be null for cell division (Morris and McCormick, unpublished). This mutation may identify a key region of FtsZ that is involved with protein polymerization into microtubule-like filaments (Romberg *et al.*, 2001; Scheffers *et al.*, 2001). Isolating suppressors of this mutation may lead to the discovery of the importance of this region where the mutation is located. I used site-directed and random mutagenesis to search for suppressors of this mutation. The site-directed mutagenesis involved changing the codon for an amino acid upstream of this region in the primary sequence to possibly relieve the effect of the first change. In suppressing the original mutation, division would be restored to the organism. A leucine at position 259 was changed to a valine. This position was chosen based on the crystal structure of

Methanococcus jannaschii FtsZ (Lowe and Amos, 1998) and the sequence comparison of FtsZ from *E. coli* and *S. coelicolor*. Random mutagenesis was attempted using a mutator strain, which is deficient in three repair systems, increasing the frequency of spontaneous point mutation approximately 5,000-fold. My hypothesis was that, by using a mutator strain, random suppressor mutations could be found, indicating interactions potentially important in polymerization not obvious from the monomer crystal structure. Random mutagenesis was also attempted using the chemical mutagen hydroxylamine.

Streptomyces coelicolor

S. coelicolor is a gram-positive filamentous sporulating soil bacterium and its 8.7 Mb genome has been sequenced and mapped (Bentley *et al.*, 2002; Redenbach, 1996). The life cycle of *S. coelicolor* is comparable to that of many filamentous fungi. *S. coelicolor* grows as a branching network of multinucleoid vegetative hyphae. As colonies age, an aerial mycelium consisting of multinucleoid hyphae grows away from the surface of the colony. As the aerial hyphae mature, they begin to undergo synchronous septation and form a long chain of uninucleoid cells that eventually will become uninucleoid spores (Chater, 1993). *S. coelicolor* is a well-characterized species. It is a model organism for the study of prokaryotic development and is an excellent organism to study cell division. Along with the fact that the cell division genes are dispensable for growth and viability, *S. coelicolor* also has a visual screen for division mutants. Certain *S. coelicolor* division mutants overproduce the blue-pigmented antibiotic actinorhodin on minimal glucose medium while the wild type colonies remain

lightly pigmented (McCormick *et al.*, 1994; McCormick and Losick, 1996). The mechanism by which this phenotype occurs is not known.

Division Genes

The majority of the work studying cell division genes in bacteria has been carried out in *E. coli*. There are ten genes in *E. coli* that are responsible for division: *ftsZ*, *ftsA*, *zipA*, *ftsK*, *ftsQ*, *ygbQ*, *ftsL*, *ftsW*, *ftsI*, *ftsN*, and *amiC* (Hale and deBoer, 1997; Buddelmeijer *et al.*, 2002; Bernhardt and deBoer, 2003; Lutkenhaus and Addinall, 1997; Chen and Beckwith, 2001). In *E. coli*, the majority of these genes are essential for growth and viability. Most of these genes were identified by mutations that resulted in a conditional lethal phenotype (Rothfield *et al.*, 1999). At non-permissive temperatures, these mutants display a filamentous temperature sensitive phenotype. The next sections will provide information about a few cell division genes, starting with *ftsZ*, the main focus of my thesis research.

ftsZ

FtsZ, the earliest acting cell division protein (Dai and Lutkenhaus, 1991), is encoded by *ftsZ* (Bi and Lutkenhaus, 1991). FtsZ is a structural bacterial homologue to eukaryotic tubulin (Erickson 1995; Erickson 1998; Romberg *et al.*, 2001; van den Ent, 2001). FtsZ has three identified regions. The first, a large, highly conserved N-terminal domain consisting of a six-stranded beta sheet surrounded by two and three helices on each side, required for binding and hydrolyzing GTP (Lowe and Amos, 1998; Nogales *et*

al., 1998). Next is a C-terminal domain of unknown function, and lastly a spacer region of variable size and sequence (Liu and Lutkenhaus, 1999; Lowe and Amos, 1998; Ma *et al.*, 1997). The core region of the N-terminal domain is responsible for FtsZ-FtsZ monomer interactions (Wang *et al.*, 1997).

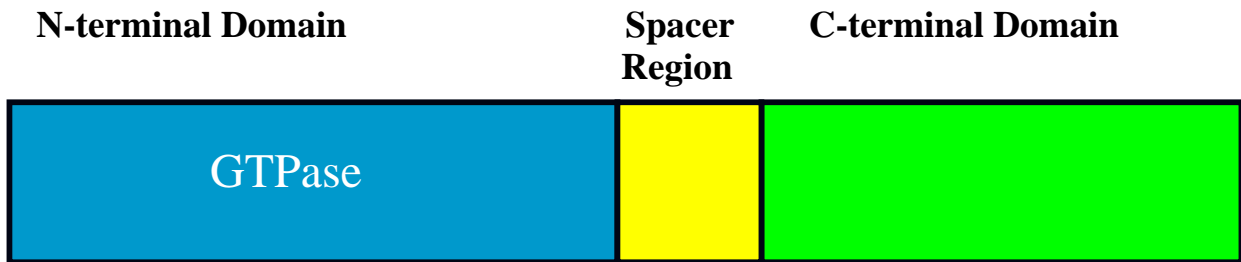


Figure 1: Domain Organization of FtsZ. Pictured above are the three key regions of FtsZ. The blue box indicates the large N-terminal domain involved in GTP-binding. The yellow box is the spacer region. The green box indicates the C-terminal domain of unknown function.

In *E. coli*, FtsZ is dispersed throughout the cytoplasm of pre-divisional cells (Lutkenhaus, 1993; Blaauwen *et al.*, 1999). Before the beginning of cell division FtsZ polymerizes to form a ring around the mid-cell known as the Z ring (Addinall and Lutkenhaus, 1996; Levin *et al.*, 2001; Rothfield and Justice, 1997). The start of septation appears to begin with the formation of the Z ring (Addinall and Lutkenhaus, 1996).

The proper concentration of FtsZ is crucial for proper cell division. Lowering the levels of FtsZ in the cell inhibits cell division (Lutkenhaus and Addinall, 1997). If there is an increase in the amount of FtsZ, cell division per bacterium increases creating anucleate mini-cells resulting from additional divisions near the poles (Lutkenhaus and Addinall, 1997). Continued overproduction of FtsZ completely inhibits cell division (Bi and Lutkenhaus, 1990; Dai and Lutkenhaus, 1991).

The *sulA* and *min* systems regulate FtsZ activity (Harry and Lewis, 2003; Justice *et al.*, 2000). The *sulA* system directly inhibits FtsZ polymerization following DNA damage (Justice *et al.*, 2000) whereas the *min* system is involved in inhibiting cell division at the poles by preventing the Z-ring from forming (Shih and Rothfield, 2003).

The highly conserved very C-terminus of FtsZ is not required for FtsZ localization (Ma and Margolin, 1999), however it is vital for its interaction with FtsA and ZipA (Yan *et al.*, 2000; Liu *et al.*, 1999). A 12 amino acid deletion of this region eliminates division in *E. coli* and *Caulobacter crescentus* completely (Ma and Margolin, 1999; Din *et al.*, 1998).

Other Cell Division Proteins

Localization of the other products involved in cell division is dependent on the prior localization of FtsZ, as seen in Figure 2. Once the Z-ring is formed, FtsA and ZipA are independently recruited to the Z-ring (Hale and deBoer, 1997; Ma and Margolin, 1999). The exact function of FtsA is not known, yet it has been shown that its localization is key to cell division in *E. coli*. ZipA is proposed to act as an anchor between the membrane and the forming Z-ring (Hale and deBoer, 1997). Recently a mutation in FtsA was isolated, which completely bypasses the requirement for ZipA (Geissler *et al.*, 2003).

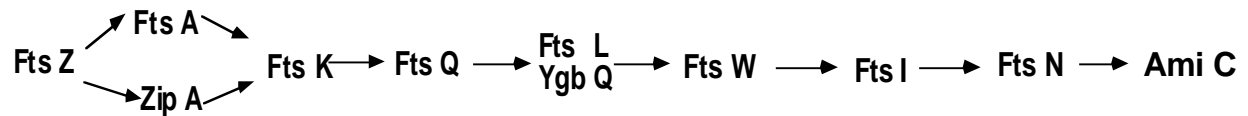


Figure 2. Assembly of Known Division Proteins to the Midcell in *E. coli*. The proteins involved in *E. coli* cell division are recruited to the site of division in a highly organized order. The localization of each protein to the site of cell division is dependent on the prior localization of the one immediately shown to the left. The process initiates with FtsZ polymerization into a Z-ring.

FtsI is also important in cell division. FtsI has been shown to localize to the site of septation (Weiss *et al.*, 1999) and synthesize the peptidoglycan at the septal cell wall (Spratt, 1975). FtsI does not localize to the Z-ring until after the majority of the other proteins have localized, including ZipA and FtsA (Weiss *et al.*, 1999; Chen and Beckwith, 2001).

FtsK is vital for cell division and appears to have two major functions: the N-terminal portion is required for cell division and the C-terminal portion is involved in the proper segregation of the chromosomes (Liu *et al.*, 1998). Each daughter cell does not receive a chromosome if FtsK is not present (Liu *et al.*, 1998).

The remaining *fts* genes have unknown functions, yet their protein products are important for localizing to the division site and each one is important for proper cell division in *E. coli* to take place.

Division in *S. coelicolor*

Homologues of the majority of the cell division genes that have been identified in *E. coli* have also been found in *S. coelicolor* and their functions are similar between the two organisms. A loss-of-function mutation in a cell division gene in *E. coli* would be lethal to the organism whereas a null mutation in an *S. coelicolor* division gene still results in a viable organism (McCormick *et al.*, 1994; McCormick, 1996). For example, *ftsZ* in *S. coelicolor* is necessary for septation. If there is a deletion of *ftsZ*, colonies will still form a mycelium but cannot sporulate because septation cannot occur. Dispensability is a distinguishing characteristic of cell division genes in *S. coelicolor*.

Structure-Directed Mutagenesis

The original mutation that is the focus of my research, *ftsZ*(A275V), is found in the C-terminal domain of FtsZ. The mutation was isolated from mutagenized *S. coelicolor* spores (J. Morris, Master's Thesis) and causes a block in division. This was a surprise because an alanine to valine change is a conservative change. I hypothesized that the reason for the block in division was due to steric hinderance caused by the larger side chain.

Inspection of the crystal structure of FtsZ_{Mj} shows that there are additional amino acids located in this region that can have potential interaction with *ftsZ*(A275V). The residue affected by the mutation is found on the backside of alpha helix HC3, located in the C-terminal domain, (boxed region pictured in the upper left of Figure 3).

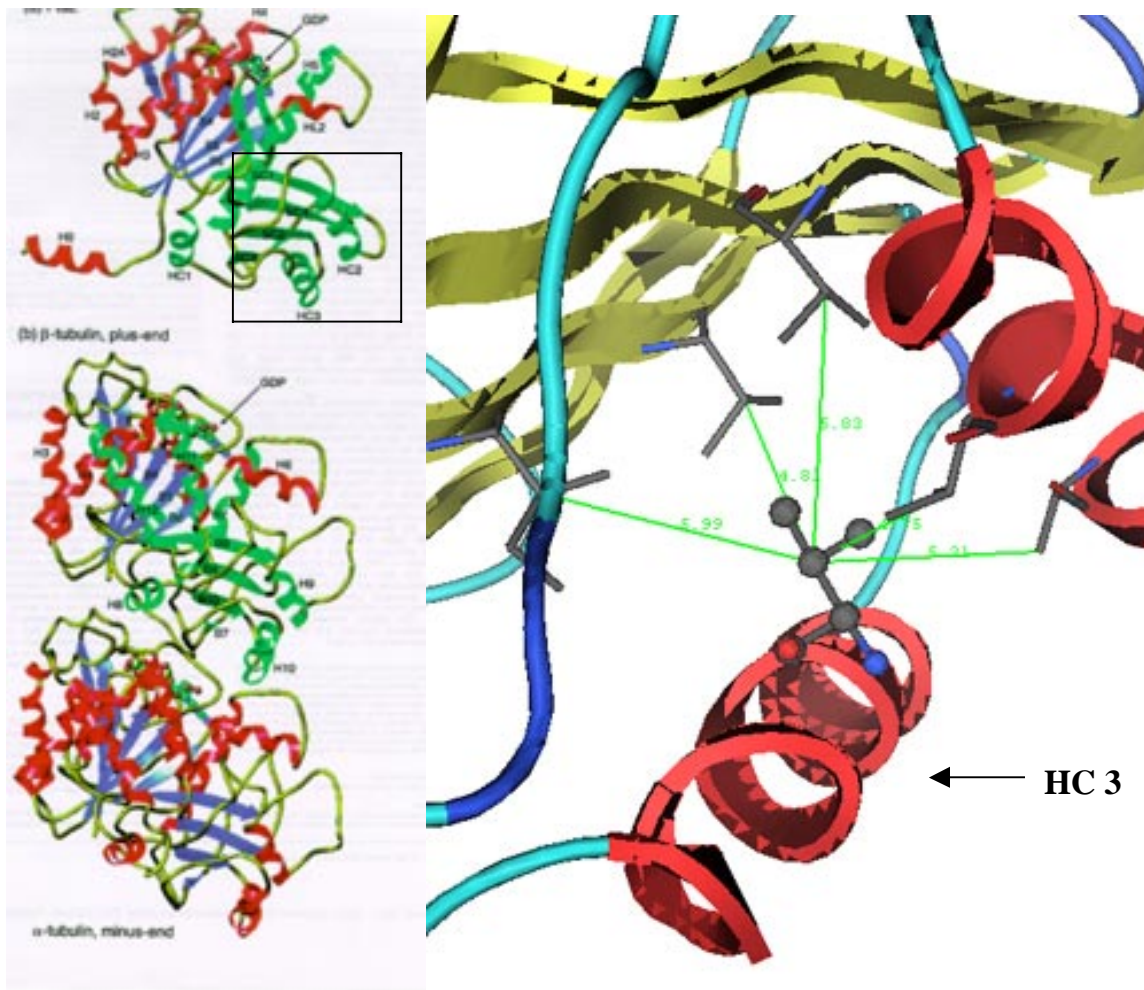


Figure 3. Crystal Structure of FtsZ from *Methanococcus jannaschii* and $\alpha\beta$ tubulin dimer. The figure on the top left shows a FtsZ monomer (Lowe and Amos, 1998). The boxed region is enlarged on the right. The figure on the bottom left shows the structure of an $\alpha\beta$ tubulin dimer (Lowe and Amos, 1998). The figure on the right, provided by J. Madura (Dept of Chemistry and Biochemistry, Duquesne University), is a version of the region of FtsZ *M. jannaschii* containing the altered amino acid (275; ball and stick) in *S. coelicolor* FtsZ and the five amino acids within distance (~ 5 Å) to interact (stick). (PDB accession code 1FSZ for *M. jannaschii*)

This domain has no known function. The C-terminal domain of FtsZ is a presumably an important region but the function is not known. There are five side chains predicted to be close enough to directly interact with the side chain of A275V in *S. coelicolor* FtsZ (Valine in *M. jannaschii* FtsZ in Figure 3). All six amino acids are not located on the same helix. The side chains are located on two different alpha helices and two different beta strands (Figure 3, right).

When comparing the interacting amino acids found in the enlarged region of FtsZ depicted in Figure 3, four of the six are identical between FtsZ of *E. coli* and *S. coelicolor* (Table 1, depicted in black). In *ftsZ*(A275V) an alanine was switched to a valine and that blocks division in *S. coelicolor*. At a comparable position in *E. coli*, the wild-type is a valine (Table 1, depicted in red). After examining the crystal structure of FtsZ and analyzing the locations of these amino acids (Figure 3), I hypothesized that the change from the alanine to valine caused steric hindrance. Even though they are both relatively small hydrophobic amino acids, the change could have a major effect on any of the following FtsZ activities: polymerization, GTP-binding, GTPase activity, or depolymerization (Scheffers *et al.*, 2002; Scheffers and Driessen, 2002; Scheffers, 2001; Mukherjee and Lutkenhaus, 1998; Mukherjee *et al.*, 2001; Lu *et al.*, 2001). My aim was to change the last remaining amino acid in this region in FtsZ of *S. coelicolor* (labeled as chemically conserved in Table 1) so that all six are identical to the wild type sequence of *E. coli* FtsZ. Since all six amino acids will be the same as another wild-type FtsZ, perhaps function would be restored to the *S. coelicolor* protein. Possibly, changing this

amino acid will relieve any putative steric problem, thus allowing the organism to divide normally.

Table 1: Comparison of Amino Acids between FtsZ of *E. coli* and *S. coelicolor* at Positions Comparable to those Identified in the Crystal Structure of *M. jannaschii*

<i>E. coli</i>	<i>S. coelicolor</i>	Comparison
Alanine 236	Alanine 234	Identical
Alanine 240	Alanine 238	Identical
Valine 262	Leucine 259	Chemically conserved
Isoleucine 264	Isoleucine 261	Identical
Valine 278	Alanine 275	Identical after A275V mutation
Valine 308	Valine 305	Identical

Random Mutagenesis

Another approach to finding suppressors of the *ftsZ*25 mutation was to chemically mutagenize plasmid DNA containing *ftsZ*(A275V) and isolate mutants that will restore division to the cell. I used hydroxylamine (NH₂OH) which deaminates cytosine residues and the deaminated C mispairs with adenine, resulting in G:C to A:T transitions. Using NH₂OH is a very effective way to mutagenize the G+C rich *S. coelicolor* DNA *in vitro* (Keiser *et al.*, 2000; Hopwood, 1999).

A second approach to random mutagenesis, a genetic approach, involved the use of a mutator strain. The strain is deficient in three repair systems (*mutS mutD mutT*) resulting in a high frequency of point mutations. *mutS* is deficient in mismatch repair, *mutD* in the exonuclease activity of DNA Polymerase III, and *mutT* which is unable to hydrolyze 8-oxod GTP. By using a mutator strain, I hoped to find a whole host of suppressors throughout the *ftsZ* gene that restore division to *ftsZ*(A275V).

METHODS

Bacterial Strains and Plasmids

E. coli strains used in this study are listed in Table 2. Strain LL308 was used for plasmid propagation. The *dam*⁻ *dcm*⁻ strain ER²-1 was used for preparation of unmodified DNA for *S. coelicolor* transformation. ET12567/pUB307 was used as a donor for mating *E. coli* with *S. coelicolor*. All *S. coelicolor* strains used are derivatives of the wild-type strain A3(2) and are found in Table 3. The prototrophic SCP1⁻ SCP2⁻ strain M145 was the parental strain of HU133 (Δ *ftsZ*). Plasmids were introduced by transformation into the multiple auxotrophic strain 2709 before being conjugated to M145 and HU133. Plasmids used or created in this study are listed in Table 4.

Media and Growth Conditions

E. coli strains were grown at 37°C on solid agar or liquid Luria broth. Antibiotic concentrations used were ampicillin at 100 µg/ml, carbenicillin at 100 µg/ml, and apramycin 50 µg/ml except that apramycin was used at 30 µg/ml for the strain ET12567/pUB307 containing pSET152 and its derivatives.

Strains of *S. coelicolor* were grown on either R2YE agar and minimal medium (Keiser *et al.*, 2000) with 0.5% glucose. Liquid cultures of *Streptomyces* were grown in YEME (Keiser *et al.*, 2000). Antibiotic concentrations used for *S. coelicolor* were thiostrepton at 50 µg/ml in plates and 10 µg/ml in liquid, neomycin at 10 µg/ml and apramycin at 25 µg/ml.

Table 2: *E. coli* Strains Used in This Study

Strain	Genotype	Source or Reference
LL308	$\Delta(\text{pro-lac}) \text{recA nalA supE thi/F}^+$	Zengel <i>et al.</i> , 1980
ER ² -1	$\text{pro}^+ \text{lacI}^q \text{lacZ}\Delta\text{M15}$	McCormick, unpublished
ET12567	$\text{ara-14 leuB6 thi-1 fhuA31 lacY1 tsx-78 galK2 galT22 supE44 hisG4 rspL36 xyl-5 mtl-1 dam-13::Tn9 dcm6 mcrB1 mcrA/F}^+ \text{proAB}^+ \text{lacI}^q \text{lacZ}\Delta\text{M15 zzf::Tn5(kan}^R) \text{dam-13::Tn9 dcm-6 hsdM hsdR recF143 zjj201::Tn10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 glnV44 F}^- \text{RP6::kan}^R \text{RP4oriT}$	MacNeil <i>et al.</i> , 1992
ET12567/ pUB307		Flett <i>et al.</i> , 1997
XL-1 Blue	$\text{recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac/F}^+ \text{proAB lacZ}\Delta\text{M15 Tn10 (tet}^R)$	Stratagene
XL-1Red	$\text{endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT:: Tn10 (Tet)}$	Stratagene
XL-10 Gold	$\Delta(\text{mcrA})183 \Delta(\text{mcrCB-hsdSMR-mrr})173 \text{endA1 supE44 thi-1 recA1 gyrA96 relA1 lac The/F}^+ \text{proAB lacI}^q\text{Z}\Delta\text{M15 Tn10 (tet}^R) \text{Amy Cam}^R$	Stratagene

Table 3: *S. coelicolor* Strains Used in This Study

Strain	Genotype	Source or Reference
M145	Prototrophic SCP1 ⁻ SCP2 ⁻	Hopwood <i>et al.</i> , 1985
HU133	$\Delta\text{ftsZ::aphI}$ (M145 background)	McCormick <i>et al.</i> , 1994
2709	$\text{proA1 hisA1 argA1 cysD18 uraA1 strA1 SCP1}^- \text{SCP2}^-$	Hopwood <i>et al.</i> , 1985

Table 4: Plasmids Used in This Study

Plasmid	Description ^a	Reference or Source
pBluescriptII pCR2.1	Commercial cloning vector; <i>amp</i> ^R PCR-Product “TA” cloning vector; <i>amp</i> ^R	Stratagene Invitrogen
pRSETB pSET152	Commercial cloning vector; <i>amp</i> ^R Mobilizable integrating vector for <i>Streptomyces</i> ; <i>apra</i> ^R	Invitrogen Bierman <i>et al.</i> , 1992
pJR125	pBluescript derivative with <i>S.</i> <i>coelicolor</i> <i>ftsZ</i> and native promoters	McCormick and Losick, 1996
pJRM10	Bifunctional derivative of pIJ922 and pBluescriptII; <i>tsr</i> ^R , <i>amp</i> ^R (Self- transmissible in <i>S. coelicolor</i>)	McCormick and Losick, 1996
pJRM12	pJRM10 with <i>S. coelicolor</i> <i>ftsZ</i> ⁺	McCormick and Losick, 1996
pJS21	Plasmid containing a His-tagged version of <i>ftsZ</i> ⁺	Schwedock <i>et al.</i> , 1997
pJWM31	pCR2.1 derivative with <i>ftsZ</i> (A275V) from strain Hg25 (PCR product)	J. Morris, Master’s Thesis
pCMC1	pCR2.1 derivative with <i>S. coelicolor</i> <i>ftsZ</i> ⁺ (PCR product)	This study
pCMC2	pCR2.1 derivative with <i>S. coelicolor</i> <i>ftsZ</i> (L259V) (PCR product)	This study
pCMC5	pCR2.1 derivative with <i>S. coelicolor</i> <i>ftsZ</i> (L259V, A275V) (PCR product with multiple additional mutations)	This study
pCMC6	pRSETB derivative containing a 1.2 kb <i>EcoR</i> I- <i>Sac</i> I fragment from pCMC1(<i>Pvu</i> II site in <i>ftsZ</i> is now unique)	This study
pCMC7	pCMC6 digested with <i>Asc</i> I- <i>Pvu</i> II containing <i>Asc</i> I- <i>Pvu</i> II fragment of pCMC5 [<i>ftsZ</i> (L259V, A275V)]	This study
pCMC8	pJR125 digested with <i>Asc</i> I- <i>Pvu</i> II containing <i>Asc</i> I- <i>Ecl</i> 136 II fragment of pCMC2 [<i>ftsZ</i> (L259V)]	This study
pCMC9	pJR125 digested with <i>Asc</i> I- <i>Pvu</i> II containing <i>Asc</i> I- <i>Ecl</i> 136 II fragment of pCMC7 [<i>ftsZ</i> (L259V, A275V)]	This study
pCMC10	pJR125 digested with <i>Asc</i> I- <i>Pvu</i> II containing <i>Asc</i> I- <i>Ecl</i> 136 II fragment of pCMC7 [<i>ftsZ</i> (L259V, A275V)]	This study

Table 4 continued

pCMC11	pJR125 digested with <i>Asc</i> I- <i>Pvu</i> II containing <i>Asc</i> I- <i>Ecl</i> 136 II fragment of pJWM31 [<i>ftsZ</i> (A275V)]	This study
pCMC12	pCMC9 derivative containing 24 kb <i>Eco</i> R I- <i>Bam</i> H I fragment from pJRM10 [<i>ftsZ</i> (L259V, A275V)]	This study
pCMC13	pCMC8 derivative containing 24 kb <i>Eco</i> R I- <i>Bam</i> H I fragment from pJRM10 [<i>ftsZ</i> (L259V)]	This study
pCMC14	pSET152 digested with <i>Eco</i> R V - <i>Eco</i> R containing <i>Acc</i> 65 I ⁰ - <i>Eco</i> R I fragment of pCMC9 [<i>ftsZ</i> (L259V, A275V)]	This study
pCMC15	pSET152 digested with <i>Eco</i> R V- <i>Eco</i> R I containing <i>Acc</i> 65 I ⁰ - <i>Eco</i> R I fragment of pCMC11 [<i>ftsZ</i> (A275V)]	This study
pCMC16	pSET152 digested with <i>Eco</i> R V- <i>Eco</i> R I containing <i>Acc</i> 65 I ⁰ - <i>Eco</i> R I fragment of pCMC8 [<i>ftsZ</i> (L259V)]	This study
pCMC17	pJS21 digested with <i>Asc</i> I and <i>Sac</i> I containing <i>Asc</i> I- <i>Sac</i> I fragment of pCMC2 [<i>his</i> ₆ - <i>ftsZ</i> (L259V)]	This study
pCMC18	pJS21 digested with <i>Asc</i> I and <i>Sac</i> I containing <i>Asc</i> I- <i>Sac</i> I fragment of pCMC7 [<i>his</i> ₆ - <i>ftsZ</i> (L259V, A275V)]	This study
pCMC19	pJR125 digested with <i>Asc</i> I and <i>Eco</i> R V containing <i>Asc</i> I- <i>Ecl</i> 136 II fragment of pCMC1 (<i>ftsZ</i> ⁺)	This study
pCMC20	pCMC9 derivative containing 24 kb <i>Eco</i> R I- <i>Bam</i> H I fragment from pJRM10 [<i>ftsZ</i> (L259V,A275V)]	This study
pCMC21	pCMC8 derivative containing 24 kb <i>Eco</i> R I- <i>Bam</i> H I fragment from pJRM10 [<i>ftsZ</i> (L259V)]	This study
pCMC22	pSET152 digested with <i>Eco</i> R V- <i>Eco</i> R I containing <i>Acc</i> 65 I ⁰ - <i>Eco</i> R I fragment of pCMC19 [<i>ftsZ</i> ⁺]	

^aRestriction enzyme sites with a superscript 0 (i.e., *Acc* 65 I⁰) were rendered blunt by treatment with Klenow fragment of DNA Pol I.

SFM (soy flour-mannitol) plates containing 10 mM MgCl₂ were used for interspecies mating between *E. coli* and *S. coelicolor* (Hopwood, 1999). Antibiotic concentrations used were apramycin at 50 µg/ml to select for *S. coelicolor* transconjugants and nalidixic acid at 25 µg/ml to counter select against the *E. coli* donors. Transconjugants were also restreaked on minimal medium with 0.5% glucose and apramycin at 25 µg/ml.

DNA Techniques

Plasmid DNA was prepared from *E. coli* using the Quick Spin Miniprep Kit (QIAGEN). The Gel Extraction Kit (QIAGEN) was used to purify DNA fragments from agarose gels. DNA restriction and modifying enzymes were used according to manufacturer's directions. After restriction digestion, phenol and chloroform were used to extract protein from DNA. DNA was recovered by ethanol precipitation. Vector restriction fragments were dephosphorylated using Calf-Intestinal Phosphatase (CIP) at 37°C for 1 hour. Products from ligation reactions were used to transform XL-1 Blue, LL308, or XL-10 Gold competent cells.

Ligation-Mediated PCR

PCR was used to introduce a mutation in *ftsZ* at codon 259, changing a leucine to a valine in an *ftsZ*⁺ fragment cloned in pCMC1 as template, resulting in pCMC2. The same mutagenesis was done to a mutant version of *ftsZ*, *ftsZ*²⁵, that already contained a

mutation, A259V, resulting in pCMC5. The template used was pJWM31. The strategy required three separate PCR reactions as shown in Figure 4. The first two PCRs were performed with templates pCMC1 and pJWM31 using the following oligonucleotide sequences: *ftsZ*750 (5'-CAGCACGCCGCGGGCGCCGTC GAT-3') together with 31Z95 (5'-CGACGTGGCTAGCGCACCGCAGAACTACCTC-3') and *ftsZ*775L259V (5'-GTCTCCATCTCCGGCGGCTCCGACCTC-3') together with 19Z95 (5'-GCCGCCGAGGTTGAGCTCC-3'). The reactions were incubated in a Biometra T1 Thermocycler [(96°C for 1', 65°C for 1', 72°C for 2') repeat 30 times, 72°C for 10'] with a proofreading DNA polymerase (*Pfx* DNA polymerase, 2.5 U; Invitrogen). This caused the *ftsZ* gene to be amplified in two adjacent parts: approximately a 5' 700 bp fragment and a 3' 500 bp fragment. Both fragments were gel purified and a kinase reaction was performed, phosphorylating both fragments. Each kinase reaction was done separately but then both products were pooled and ethanol precipitated to concentrate the DNA. The DNA was resuspended in 3 µl 1X DNA ligase buffer, and T4 DNA ligase (1 U; Invitrogen) and incubated at room temperature for 15 minutes. The reaction was terminated by an incubation at 65°C for 15 minutes. Another ethanol precipitation was performed and the DNA was resuspended in 28 µl of water. PCR was then done using the entire ligation mixture in a 50 µl reaction volume, as template, *Pfx* DNA Polymerase (Invitrogen) and outside oligonucleotide sequences (31Z95 and 19Z95). The amplification program was the same as described as above.

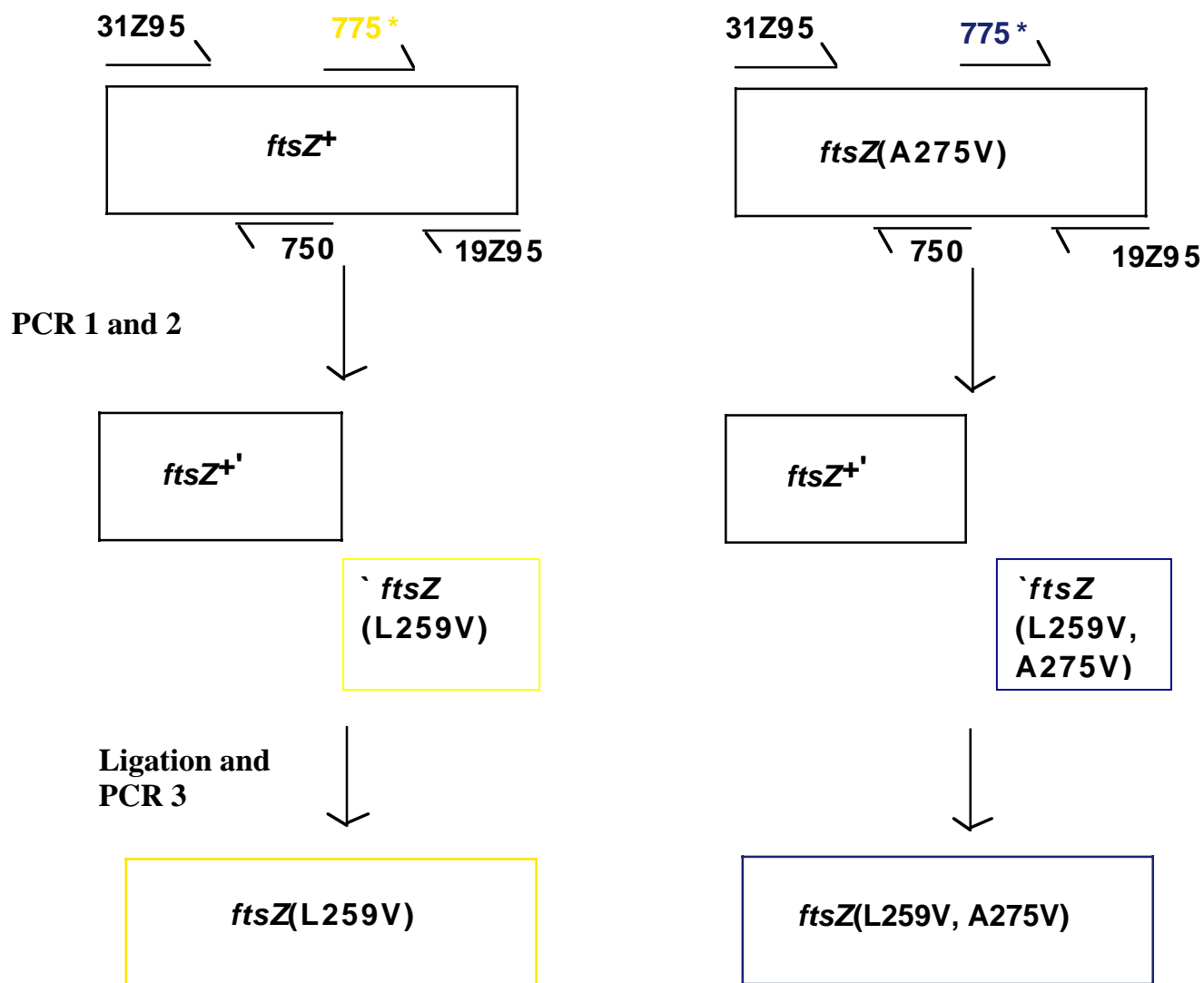


Figure 4. Strategy for Mutagenesis using Ligation-Mediated PCR. The figure is a representation of the protocol followed to create both the *ftsZ*(L259V) mutation and the *ftsZ*(L259V, A275V) mutation. Top) The labeled boxes indicate the template used in separate PCR reactions using two sets of oligonucleotide sequences. Oligonucleotide 775* contains a point mutation to change codon 259 from a leucine to a valine. The resulting PCR products were ligated together using T4 DNA ligase. The ligation mixture was then used as template for a third PCR reaction using the two outside primers. The resulting PCR products contained the introduced mutations.

To add A-overhangs needed for cloning, *Taq* DNA polymerase (5 U; Invitrogen) was added and incubated on the Biometra T1 Thermocycler [(96°C for 1', 68°C for 1' 72°C for 2') repeat 2 times, 72°C for 10']. The 1.2 kb *ftsZ* fragment was gel purified and then cloned into pCR2.1TOPO (Invitrogen). Candidates were verified by sequencing.

DNA Sequencing

The *ftsZ* gene in pCMC2[*ftsZ*(L259V)] and pCMC5[*ftsZ*(L259V, A275V)] were sequenced using *ftsZ*651 (5'-CTTCAGCCAGTACAGGCTC-3') in order to ensure that the desired mutation(s) were present in *ftsZ*. The gene also needed to be sequenced in its entirety to ensure that the only mutations were the ones that I introduced. Two oligonucleotides sequences, M13R and M13F, anneal to the vector and were used to sequence the 5' and 3' ends of the gene. The entire *ftsZ* gene was sequenced using the following custom primers: *ftsZ*upGBox (5'-GAGGAGATCGAGGAGGTCCT-3'), *ftsZ*438 (5'-GGCCAACCAGGCCGAGGAC-3'), *ftsZ*493 (5'-GTCGTTGGGGATGACGA-3'), *ftsZ*651 (5'-CTTCAGCCAGTACAGGCTC-3'), *ftsZ*872 (5'-CCGAGGCCAACATCATCTTCG-3'), and *ftsZ*923 (5'-TCACGGTGACCCGCACCT-3'). All reactions used the Thermo Sequenase Big Dye-Terminator cycle kit (ABI). Sequences were determined using a Genetic Analyzer (ABI Prism 310) and compared against the wild-type sequence of the *S. coelicolor ftsZ* gene (GenBank accession number U10879) using the programs AssemblyLine and BLAST. pCMC2 was correct and only contained the introduced mutation. Several candidates for

pCMC5 contained the introduced mutations along with additional mutations. The one chosen for further work contained five additional mutations.

Construction of pCMC6 and pCMC7

The DNA insert of pCMC5, isolated following ligation-mediated PCR, contained five additional mutations and could not be used directly for additional cloning steps. Therefore, the second step in this process was to excise a small fragment from pCMC5, which encompassed the L259V and A275V-containing portion of *ftsZ*, and replace a corresponding section in a wild-type copy of *ftsZ*. A *Pvu* II site is located in *ftsZ* just downstream of the region containing both desired mutations. In order to make this *Pvu* II site unique, the insert of pCMC1 was moved to pRSETB using *Eco*R I and *Sac* I, which has no *Pvu* II sites. Candidates for the pRSETB derivative were verified using an *Eco*R I and *Bam*H I double digestion. The resulting plasmid was named pCMC6 and now contained a unique *Pvu* II site in *ftsZ*⁺ (Table 4).

The next step was to excise the *ftsZ*(L259V, A275V) fragment from pCMC5 and insert it into a wild-type *ftsZ* in pCMC6. Both pCMC5 and pCMC6 were digested with *Asc* I and *Pvu* II and the appropriate fragments were gel purified and ligated. Candidates for the resulting plasmid were verified by sequencing with primer *ftsZ*651. A plasmid containing both correct mutations was named pCMC7 and contained *ftsZ*(L259V, A275V) (Table 4).

Construction of pCMC8, 9, 10, 11, and 19

To observe the phenotypes of the mutations introduced into *ftsZ*, I needed to clone the promoterless *ftsZ* downstream of the natural *S. coelicolor* promoters. This was done using the promoter-containing fragment of plasmid pJR125. pCMC1[*(ftsZ⁺)*], pCMC2[*(ftsZ(L259V))*], pCMC7[*(ftsZ(L259V, A275V))*], and pJWM31[*(ftsZ(A275V))*] were digested with *Asc* I and *Ecl*136 II. pJR125 was digested with *Asc* I and *Eco*R V and dephosphorylated. Appropriate fragments were gel purified and ligated. Candidates were verified with a double digestion using *Asc* I and *Sma* I. Plasmids were also verified for their appropriate mutations by sequencing using primer *ftsZ*651. Plasmids were named pCMC8, 9, 10, and 11 (Table 4).

Construction of pCMC12, 13, 20 and 21

Now that the *ftsZ(L259V)* and *ftsZ(L259V, A275V)* genes were placed downstream of natural promoters in an *E. coli* plasmid, pCMC8 and pCMC9 needed an origin of replication and selectable marker (thiostrepton resistance) for *S. coelicolor*. The pIJ922-derived fragment of pJRM10 was used. pCMC8, pCMC9, and pJRM10 were digested with both *Eco*R I and *Bam*H I. The purified pJRM10 insert fragment (24 Kb) was ligated with the gel purified vector fragments of pCMC8 and pCMC9. Candidates were verified with a double digestion using *Eco*R I and *Bam*H I. Candidates were further verified by a digest with *Sac* I. Plasmids were named pCMC13 and pCMC21 and pCMC12 and pCMC20, respectively (Table 4).

Construction of pCMC14, 15 , 16 and 22

pSET152 is a site-specific integrating vector for *S. coelicolor* based on ϕ C31 *int* and *att*, and also can be conjugated from *E. coli* and *S. coelicolor*. pSET152 was digested with *EcoR* V and *EcoR* I. pCMC8[(*ftsZ*(L259V))], pCMC10[(*ftsZ*(L259V, A275V))], pCMC11[(*ftsZ*(A275V))], and pCMC19[(*ftsZ*⁺)] were individually digested with *Acc65* I and were filled in using the Klenow fragment of *E. coli* DNA Polymerase I. Subsequently all four were then separately digested with *EcoR* I. The pSET152 vector was individually ligated to the four inserts. Candidates were verified with a double digestion using *EcoR* I and *BamH* I. As a further check, single digests using *Xho* I and *Hind* III were also used. Plasmids were also checked for their appropriate mutations by sequence analysis using primer *ftsZ*651. Plasmids were named pCMC14, 15, 16, and 22 (Table 4).

Construction of pCMC17 and pCMC18

In order to facilitate the purification of altered FtsZ to measure GTPase activity and the ability to polymerize in an *in vitro* assay, *ftsZ* needed to be cloned adjacent to poly-His encoding sequence. The His-tag will allow for purification of the protein. pJS21 is a plasmid that already contains a poly-His sequence at the 5' end of *ftsZ*⁺ and has sites that can be used to replace part of the plasmid with altered *ftsZ* genes. pJS21 was digested with *Asc* I and *Sac* I. pCMC2[(*ftsZ*L259V)] and pCMC7[(*ftsZ*L259V, A275V)] were also digested with *Asc* I and *Sac* I. The vector pJS21 was

dephosphorylated and ligated to the two inserts. Candidates were verified with a double digestion using *Asc* I and *Sac* I. Candidate plasmids were also checked for their appropriate mutations by sequence analysis using primer *ftsZ651*. Sequence verified plasmids were named pCMC17 and pCMC18 respectively (Table 4). These were constructed here and will be used later by the laboratory for future experiments.

S. coelicolor* Transformation Using Plasmid DNA Isolated from *E. coli

Plasmid DNA has to be isolated from methylation-deficient *E. coli* before transforming into methyl-DNA restricting *S. coelicolor*. Therefore plasmid DNA was transformed into strain ER²-1. DNA isolated from ER²-1 derivatives was then ready for transformation into *S. coelicolor*.

In brief, *S. coelicolor* 2709 spores were inoculated in 30 ml of YEME containing 0.5% glycine and 5 mM MgCl₂ and incubated at 30°C for 36-48 hours. *S. coelicolor* mycelia were washed three times with a solution of 10.3% sucrose. The washed mycelia were then resuspended in a lysozyme solution (2 mg/ml) in P-buffer (0.5% KH₂PO₄, 3.68% CaCl₂, 5.73% TES [(Tris(hydroxymethyl) methyl]-2-aminoethanesulfonic acid, pH 7.2)], incubated at 30°C for approximately 90 minutes. The resulting protoplasts were filtered through sterile cotton and recovered by centrifugation and resuspended in 200 µl P-buffer. Plasmid DNA (pCMC12 and pCMC13) was added to 100 µl aliquots of protoplasts. The immediate addition of 500 µl of P-buffer + 25% PEG 1000 followed. The transformed protoplasts were plated on R2YE medium and incubated at 30°C. After 16-20 hours, petri plates were overlayed with thiostrepton to select for transformants.

The overlayed plates were incubated at 30⁰ C. Individual colonies were picked and restreaked three times on minimal glucose plates with thiostrepton (50 µg/ml).

Phase-Contrast Microscopy

Division phenotypes were analyzed after growth on solid medium supplemented with 0.5% glucose and thiostrepton (10 µg/ml). Cover slip lifts were made by inserting sterile cover slips into solid medium at a 45⁰ angle. The cover slips were inoculated with mechanically macerated colonies and incubated at 30⁰C for 3-4 days. Individual cover slips were removed and mounted using 20% glycerol. A Nikon Microphot-SA microscope was used for observation and cover slips were observed under 100x oil immersion. A Nikon UFX-DX camera and Kodak 100 T film was used to capture images. The negatives were scanned using a Nikon Film Scanner (LS-3000) and imported to Adobe Photoshop.

Isolation of Whole Cell Protein Extracts for Analysis

Approximately 200 colonies of plasmid-containing strains of each mutant (pCMC12, pCMC13, and pJWM34) and 100 colonies of the plasmid containing strain of wild-type were mechanically macerated in saline and grown in 30 ml of YEME containing 15% glycine and 5 mM MgCl₂ supplemented with 10 µg/ml thiostrepton. Cultures were grown, with constant agitation at 30⁰C for 40-44 hours. The mycelium were washed three times with 10.3% sucrose. Mycelial pellets were resuspended in lysis buffer (0.1 M Tris, pH8, 60 mM NaCl, 14 mM MgCl₂, 6 mM 2-mercaptoethanol)

containing proteinase inhibitor (1 mM PMSF). Sonication, using a SonicDismembrator (Fisher Scientific, Springfield, NJ), of the resuspended pellets was performed on ice with 10 second bursts at 10 watts and 20 second cooling for a total of 6 times during a 3 minute interval. The sonication extracts were centrifuged at 4°C at 12,000 X g for 10 minutes. The supernatant was collected, which contained the whole cell protein extract, and stored at -80°C for future use. Analysis was completed with two independent isolates of strains containing pCMC12 and pCMC13.

Protein Concentration Determination

The amount of total protein contained in whole cell extracts was determined using the Bradford dye-binding protein assay (Bio-Rad, Hercules, CA). Bovine serum albumin (BSA) was used as a standard.

SDS-PAGE and Western Blot Analysis

SDS-PAGE gels were run on Mini-PROTEAN® III electrophoresis systems (Bio-Rad, Hercules, CA). The 4-15 % Tris-Cl gradient polyacrylamide gels (Ready Gel; BioRad) were electrophoresed in running buffer (25 mM Tris pH 8.3, 18 mM glycine, 0.35 mM SDS) at 175 volts for 60 minutes. The molecular weight standard used was Benchmark prestained protein ladder (Invitrogen). Samples were prepared based on protein concentrations determined from the Bradford assay and 9 µg was loaded per lane. Gels were either stained with Coomassie Blue (0.125% Coomassie Blue R-250, 30%

methanol, 10% acetic acid) or transferred to PVDF membrane for immunological detection.

Proteins were transferred to PVDF membranes (Millipore) in Transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) at 100 volts for 1 hour in a transfer apparatus (BioRad) packed in ice. Membranes were blocked for 1 hour at room temperature in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) which also contained 5% (w/v) powdered milk (Food Club). Blocked Western blots were washed with TBST with no powdered milk. To detect FtsZ, the blots were incubated in TBST containing a 1:50,000 dilution of polyclonal FtsZ antibody (Schwedock *et al.*, 1997) for 60 minutes at room temperature under constant agitation. Excess antibody was removed by washing with TBST, 3 times at 7 minutes per wash. To detect the bound FtsZ antibody, secondary antibody [alkaline phosphatase-conjugated anti-rabbit IgG (Fc, Promega)] was diluted in TBST to a final concentration of 1:5,000 and added to the western blots and incubated for 60 minutes under constant agitation. Excess secondary antibody was removed by washing with TBST, 3 times at 7 minutes per wash, followed with one 30-second wash with TBS (20 mM Tris-Cl, 150 mM NaCl, pH 7.5). The blots were developed (usually 10-30 minutes) using color development substrates NBT (Nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indo phosphate) (Promega) in alkaline phosphatase buffer (100 mM Tris-Cl, 100 mM NaCl, 5 mM MgCl₂, pH 9.5). Developed blots were washed with water to terminate the reaction.

Interspecies Conjugation from *E. coli* to *S. coelicolor*

Plasmid-containing *E. coli* cultures (donor strain ET12567/pUB307) were grown overnight in LB liquid containing chloramphenicol (25 µg/ml), kanamycin (25 µg/ml), and apramycin (30 µg/ml). The overnight culture was diluted 1:100 into a liquid culture containing only apramycin (30 µg/ml) and grown to an OD₆₀₀ between 0.4 and 0.6. The cells were centrifuged and the pellet was washed twice with an equal volume of LB liquid containing no antibiotic.

Approximately 100 µl of spores of *S. coelicolor* M145 (wild-type strain) spores were mixed with 900 µl of 2XYT (Hopwood, 1999) to wash the glycerol from the spore stock and centrifuged for 5 minutes. The spore pellet was resuspended in 500 µl of 2XYT and heat-shocked at 50°C for 10 minutes and then allowed to cool. After five minutes 500 µl of mutagenized *E. coli* cells were added and centrifuged for two minutes. The supernatant was removed and the pellet resuspended in the remaining liquid. The entire resuspended pellet was plated onto one SFM plate containing 5 mM MgCl₂ and incubated at 30°C overnight. The next day the plate was overlaid with a 2 ml solution containing 25 µg/ml nalixidic acid and 30 µg/ml apramycin and incubation was continued at 30°C. Transconjugants were observed within a few days. The same protocol was used when conjugating with HU133 (Δ *ftsZ*) except that mycelial fragments were used and the heat-shock was omitted.

Hydroxylamine Mutagenesis

Hydroxylamine (NH_2OH) mutagenesis was done to both pCMC22(*ftsZ*⁺) and pCMC15[*ftsZ*(A275V)]. The protocol was previously used to isolate a mutant of *ftsZ* (Grantcharova *et al.*, 2003). Hydroxylamine (Sigma) was prepared in the following manner: 0.11 g of NH_2OH was dissolved in 1.4 ml of 4N NaOH. The 100 μl reactions containing NH_2OH were set up as follows: 20 μl phosphate-EDTA (0.5 M K_2PO_4 , 0.5 M KH_2PO_4 , 5 mM EDTA), 70 μl of NH_2OH , 5 μg DNA, and the corresponding amount of water to equal 100 μl . Control 100 μl reactions without NH_2OH were the same except that, 70 μl of 4 N NaOH instead of NH_2OH was used. Immediately 25 μl were removed (time point of zero) and placed in a mini-dialysis chamber (Pierce) to be drop dialyzed to remove the NH_2OH . The remaining 75 μl samples were incubated at 37°C. At 12, 24, and 36 hours 25 μl samples were removed and drop-dialyzed against approximately 200 ml TE buffer (10 mM Tris, 0.1 mM EDTA). The buffer was changed three times approximately every 1-2 hours. The dialyzed samples were ethanol precipitated to recover DNA.

Analysis of Mutagenesis Efficiency

Hydroxyl amine-treated DNA was transformed into *E. coli* to test the efficiency of the treatment, testing for a drop in transformation efficiency. After ethanol precipitation, DNA was resuspended in 25 μl TE buffer and 10 μl of DNA was used for transformation. A ten-fold dilution series was performed before plating 100 μl on LB medium containing

apramycin (30 µg/ml) and kanamycin (25 µg/ml). Colonies were counted following incubation at 30°C.

Genetic Mutagenesis using an *E. coli* Mutator Strain

pCMC15 was transformed into XL-1Red (*mutD mutS mutT* ; Stratagene) following the manufacturer's directions. Two independent libraries were constructed. The cells were plated on LB agar containing apramycin (30 µg/ml) and allowed to grow overnight at 30°C. Resulting colonies (approximately 200 for each library) were pooled and grown overnight in LB liquid containing apramycin (30 µg/ml). Mutagenesis occurs during these incubations. Plasmid DNA was extracted using the QIAGEN mini-prep kit. The *ftsZ* insert in the plasmid DNA was verified by doing a restriction digest using *Bam*H I and *Eco*R I. After verification, stocks were frozen as 1 ml aliquots for later use.

XL-1 Red-mutagenized DNA was transformed into ET12567/pUZ8002 and plated on LB medium containing apramycin (30 µg/ml) and kanamycin (25 µg/ml) and placed at 37 °C to grow overnight. Resulting colonies (approximately 2,500 per library) were pooled using LB liquid. The plates were washed with 2.5 mls LB liquid. After pooling, the cultures were centrifuged for 5 minutes and resuspended in 20% glycerol. One ml aliquots of the culture were stored at -80°C for future use. When used, cells were thawed on ice and vortexed to form a homogeneous mixture. Cultures containing 30 ml LB liquid with apramycin (30 µg/ml) and kanamycin (25 µg/ml) were inoculated with 30 µl of thawed stock culture (1: 1,000 dilution). The culture was grown, shaking at 37 °C.

Readings were taken every hour until an OD₆₀₀ between 0.4 and 0.6 was reached. After the appropriate OD₆₀₀ was reached, 10 ml of the culture was removed and centrifuged for 5 minutes. It was washed 2 times with 10 ml of LB to remove the antibiotics. After the final wash, it was resuspended in 1 ml of LB.

HU133 (Δ *ftsZ*) colonies were prepared by mechanically macerating approximately 12 colonies from 7-day-old plates in 500 μ l of 20% glycerol. Matings were done on SFM plates by taking the 500 μ l of HU133 mycelial fragments and mixing with 100 μ l of prepared *E. coli* culture. Plates were placed at 30 °C overnight. The next day the plate was overlaid with a 2 ml solution containing sufficient antibiotic to give a final concentration of 25 μ g/ml nalixidic acid and 30 μ g/ml apramycin and incubation was continued at 30°C. Transconjugants were observed within a few days and potential colonies of interest were restreaked onto SFM containing nalixidic acid (20 μ g/ml) and apramycin (25 μ g/ml). Colonies were screened for those with a gray aerial mycelium because spores become pigmented late in development.

Division phenotypes of potential suppressor containing strains were analyzed after growth on minimal medium supplemented with 0.5% glucose and apramycin (30 μ g/ml). The cover slips were inoculated with mechanically macerated colonies and incubated at 30°C for 3-4 days.

RESULTS

Structure-Directed Mutagenesis: *ftsZ*(L259V) and *ftsZ*(L259V, A275V)

The goal of my thesis project was to attempt to restore division to *ftsZ*(A275V), a division mutant previously isolated in the laboratory. Using the crystal structure of *Methanococcus jannaschii* FtsZ (FtsZ_{Mj}) and the comparison of primary amino acid sequence between FtsZ_{Mj}, *E. coli* FtsZ (FtsZ_{Ec}), and *S. coelicolor* FtsZ (FtsZ_{Sc}) a structure-directed change was chosen at codon 259, changing a leucine to a valine, anticipating that this would restore function. This change would make the region in FtsZ_{Sc} identical to a wild-type protein FtsZ_{Ec}. I also wanted to see if the leucine to valine change alone would affect division.

The structure-directed changes were created by ligation-mediated PCR using the wild-type gene and *ftsZ*(A275V) as templates and the PCR products were cloned. The altered *ftsZ* was sequenced in its entirety ensuring that the induced mutation was the only mutation present. When candidates for the double mutant version of the *ftsZ* gene were sequenced, the PCR reaction created additional mutations in the 15 clones characterized. To eliminate the additional mutations, restriction sites were used to excise the portion containing the two induced mutations and these were cloned into a wild-type copy of the *ftsZ* gene.

After additional cloning steps, bifunctional vectors containing the altered genes with natural promoters were constructed and were called pCMC12 and pCMC13. Once pCMC13 [*ftsZ*(L259V)] and pCMC12 [*ftsZ*(L259V, A275V)] were created, the plasmids

were transformed into the DNA methylation-deficient strain of *E. coli* because *S. coelicolor* will degrade methylated DNA. DNA prepared from this strain was transformed into *S. coelicolor* auxotrophic strain 2709. Transformants were picked and restreaked. From 2709, pCMC13 was mated into M145 (*ftsZ*⁺) and HU133 (Δ *ftsZ*) and the resulting transconjugants were selected on minimal medium containing glucose and thiostrepton. pJRM12, which contains a wild-type copy of *ftsZ*, served as a positive control, and pJRM10, which is vector only, served as a negative control. In addition pJWM34, which contains *ftsZ*(A275V), was used for comparison. After strains were obtained, the effects of *ftsZ*(L259V) and *ftsZ*(L259V, A275V) on division were viewed under phase-contrast microscopy.

Phase-Contrast Microscopy Examination of *ftsZ*(L259V) and *ftsZ*(L259V, A275V)

Sporulation is an easy assay for division. By viewing aerial filaments under phase-contrast microscopy, the division phenotype of a strain can be easily determined. Phase-contrast microscopy was used to examine the phenotypes displayed by pCMC13 [*ftsZ*(L259V)], pCMC12 [*ftsZ*(L259V, A275V)], pJRM12 (*ftsZ*⁺), pJRM10 (vector only) and pJWM34 [*ftsZ*(A275V)] mated into both HU133 (Δ *ftsZ*) (Figure 5A) and M145 (*ftsZ*⁺) (Figure 5B). In a strain deleted for *ftsZ*, the Z-null (HU133), the only source of *ftsZ* is the *ftsZ* on the plasmid. pJRM12 complemented the Z-null, resulting in wild-type levels of sporulation, as demonstrated in the first panel of Figure 5A. pJRM10, as seen in the second panel of Figure 5A was unable to complement the Z-null resulting in all aerial

filaments. pJWM34 also behaved as previously observed (J. Morris, Master's Thesis). *ftsZ*(A275V) is unable to support division alone, seen in the third panel of Figure 5A.

The next groups of transconjugants analyzed were those of pCMC13 and pCMC12 in HU133 ($\Delta ftsZ$). For the HU133 transconjugants, when the only source of FtsZ is the one that contains either a single or double mutation, cells were unable to divide (fourth and fifth panels of Figure 5A). Aerial filaments of transconjugants were similar to those of $\Delta ftsZ$ mutant (not shown; McCormick *et al.*, 1994).

As anticipated, M145 transconjugants containing pJRM12 (*ftsZ*⁺) sporulated at wild-type levels, as seen in the first panel of Figure 5B. pJRM10 (vector only) sporulated at wild-type levels, as seen in the second panel of Figure 5B. pJWM34 behaved as previously observed (J. Morris, Master's Thesis) and acted as a dominant-negative. The next groups of transconjugants to be viewed were those of pCMC13 and pCMC12 in M145 (*ftsZ*⁺). The M145 transconjugants, which contain a chromosomal wild-type copy of *ftsZ* as well as *ftsZ*(L259V) or *ftsZ*(L259V, A275V) were also unable to divide, indicating that both act as dominant-negatives (fourth and fifth panels of Figure 5B). Aerial filaments of transconjugants were similar to those of $\Delta ftsZ$ mutant.. The copy number of the vector is a 1:1 ratio which could influence the phenotypes displayed.

Testing Stability of FtsZ(L259V) and FtsZ(L259V, A275V)

Both strains expressing altered FtsZ, which contain the induced mutations are unable to divide. Both altered versions of *ftsZ* also act as dominant-negative mutations when expressed as an additional copy in a cell with a chromosomal wild-type copy of

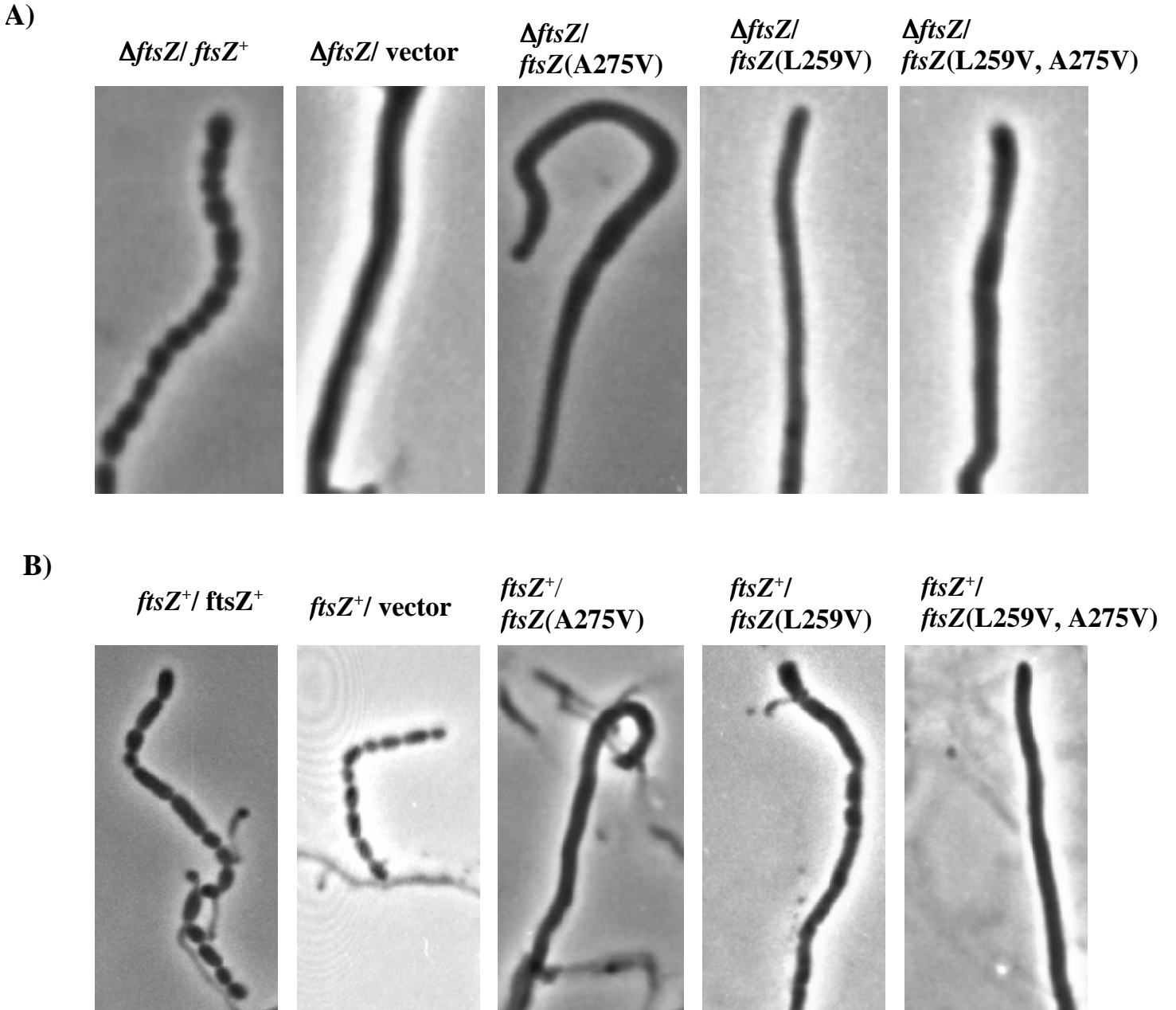
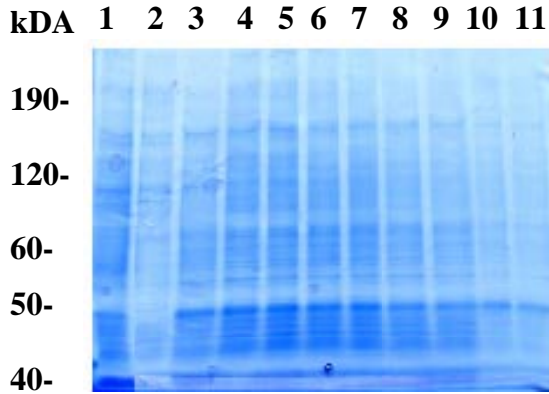
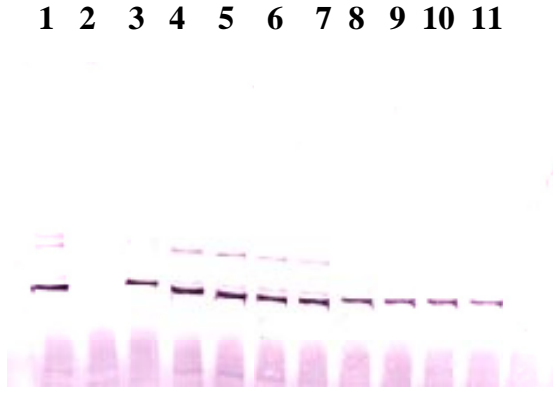


Figure 5. Phenotypes Expressed by *ftsZ*(L259V) and *ftsZ*(L259V, A275V) in $\Delta ftsZ$ and *ftsZ*⁺ Strains. Shown are phase-contrast micrographs of aerial filaments taken after 5 days growth on minimal glucose agar with thiostrepton (10 $\mu\text{g}/\text{ml}$). A) The five panels show transconjugants in HU133 ($\Delta ftsZ$). The plasmid encoded allele is the only source of FtsZ. B) The five panels show transconjugants in M145 (*ftsZ*⁺). The plasmid allele is expressed in the presence of a wild-type copy of *ftsZ*. Both strain backgrounds contain pJRM12 [*ftsZ*⁺], pJRM10 (vector only), pJWM34 [*ftsZ*(A275V)], pCMC13 [*ftsZ*(L259V)], and pCMC12 [*ftsZ*(L259V, A275V)].

A)



B)



Lane 1: $\Delta ftsZ / ftsZ^+$
 Lane 2: $\Delta ftsZ$
 Lane 3: $\Delta ftsZ / A257V$
 Lane 4: $\Delta ftsZ / L259V$ (Sample #1)
 Lane 5: $\Delta ftsZ / L259V$ (Sample #1)
 Lane 6: $\Delta ftsZ / L259V$ (Sample #2)
 Lane 7: $\Delta ftsZ / L259V$ (Sample #2)
 Lane 8: $\Delta ftsZ / L259V, A275V$ (Sample #1)
 Lane 9: $\Delta ftsZ / L259V, A275V$ (Sample #1)
 Lane 10: $\Delta ftsZ / L259V, A275V$ (Sample #2)
 Lane 11: $\Delta ftsZ / L259V, A275V$ (Sample #2)

Figure 6. Altered Versions of FtsZ are Stable and Accumulate to Levels Similar to that of Wild-Type *in vivo*. HU133 ($\Delta ftsZ$) containing plasmids pJRM12 [$(ftsZ^+)$], pJRM10 (vector only), pJWM34 [$ftsZ(A275V)$], pCMC13 [$ftsZ(L259V)$], and pCMC12 [$ftsZ(L259V, A275V)$] were grown in liquid YEME and a total protein extract was prepared. The plasmid encoded allele is the only source of FtsZ. A) Equal amounts (9 μ g) were fractionated on an SDS-PAGE 4 %-12 % gradient gel and stained with Coomassie blue. B) Shown is the Western blot of the duplicate gel to analyze protein accumulation *in vivo*. Two independent samples for strains containing pCMC12 and pCMC13 were analyzed. Bands seen at approximately 49 KDa represent FtsZ. (Migration of molecular weight standards are labeled on the left for both gels). FtsZ (L259V) protein may appear to migrate faster but the same samples fractionated on a different day did not (data not shown).

ftsZ⁺. A reason that division does not occur when it is the only copy in the strain deleted for *ftsZ* could be attributed to the fact that the altered versions of FtsZ alters the folding of the protein and the protein does not accumulate in the cell. It is important to determine that the proteins are stable *in vivo* and that they accumulate at levels equal to those of the wild-type protein. SDS-PAGE and Western blot analysis was used. First I grew cells and obtained protein extracts. A Bradford Assay was carried out to quantify the amount of total protein in each whole cell extract sample so equal amounts of total protein would be loaded in each lane. All protein samples were loaded on two SDS-PAGE gels to show the reproducibility of the analysis. One gel, stained with Coomassie blue, was used to make sure the amount of protein loaded per lane was approximately equal, as seen in Figure 6A. The second gel was transferred to a PVDF membrane and overlayed with anti-FtsZ antibody to check for stability of the protein when compared to the wild-type. As seen in Figure 6B, all proteins are stable and accumulate to levels approximately equal to that of the wild-type.

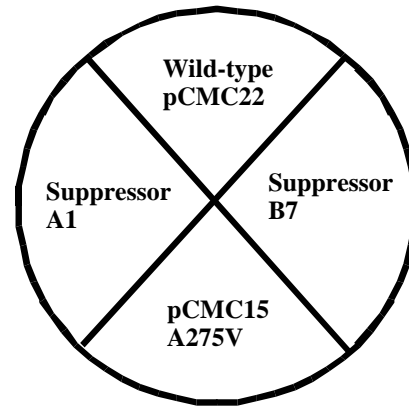
Random Mutagenesis of *ftsZ*(A275V) Using a Mutator Strain

The second part of my thesis included using a mutator strain to induce random suppressor mutations of *ftsZ*(A275V). This was accomplished by introducing pCMC15, bearing *ftsZ*(A275V), into a mutator strain of *E. coli* (XL-1Red) and then mating the resulting library into *S. coelicolor* to screen for suppressor mutations that restore division. XL-1 Red is deficient in three genes of the primary DNA repair pathways: the *mutS* , *mutD* , and *mutT* , increasing the random mutation rate by 5000-fold. During the time

that pCMC15 was propagated in XL-1Red, mutations should occur randomly throughout the gene. pCMC15 contains an *int* gene and *att* site for ϕ C31, which allows integration into the chromosome, along with *oriT*, which makes it ready for immediate mating from *E. coli* to *S. coelicolor*. The library of mutagenized plasmid isolated from XL-1 Red was transformed into ET12567/ pUZ8002, which provides plasmid transfer functions and was mated with HU133 (Δ *ftsZ*) because in HU133 the only copy of *ftsZ* located on the plasmid. The matings were carried out on Soy Flour Mannitol (SFM) plates selecting transconjugants with apramycin and counterselecting *E. coli* with nalixidic acid. Restoration of division can be screened directly on this medium. Colonies that are unable to divide (i.e. Δ *ftsZ*/pCMC15) produce a white aerial mycelium on SFM plates, whereas wild-type colonies (Δ *ftsZ*/pCMC22) produce a brown/gray aerial mycelium on SFM because spores become pigmented late in development. I looked for brown/gray colonies, indicating that division had been restored to *ftsZ*(A275V).

Using the color differences displayed by both wild-type and division mutants, two possible suppressors were isolated from a screen of about 4,000 colonies (Figure 7). The plasmids containing the two possible suppressors of the original mutation were called pCMC15-A1 and pCMC15-B7. These are independent isolates obtained from the two separate libraries. Figure 7 shows how similar the aerial mycelium of the two possible suppressors are in color to the wild-type, and how distinctly different they are when compared to the starting single mutant. The colonies are also different in appearance when grown on minimal glucose. For unknown reasons, certain division

A)



B)

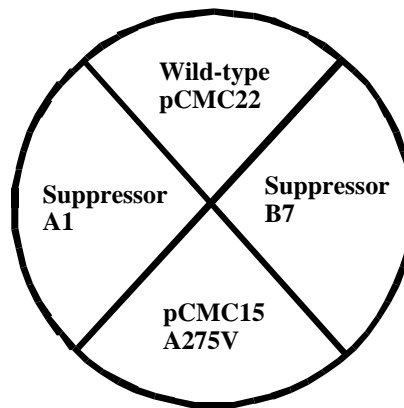
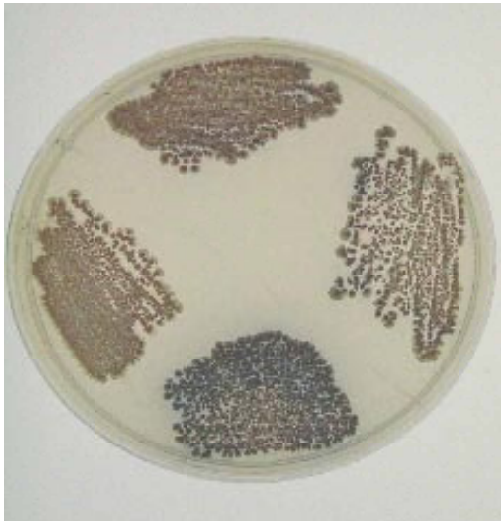


Figure 7. Two Possible Intragenic Suppressors of *ftsZ*(A275V) Identified by a Gray/white Visual Screen. All plasmids are contained in strain HU133 ($\Delta ftsZ$). Therefore the only source of FtsZ is the plasmid copy of the gene. Both plates have been incubated for 5 days of growth. A) Soy-flour mannitol plate showing the similarity of gray aerial mycelium between strains containing pCMC22 (wild-type), pCMC15-A1, and pCMC15-B7. Strain containing pCMC15 [*ftsZ*(A275V)] has a white aerial mycelium. B) Minimal glucose agar plate showing the blue-halo phenotype seen in the strain containing pCMC15 [*ftsZ*(A275V)]. Colonies of strains containing pCMC22 (*ftsZ*⁺), pCMC15-A1, and pCMC15-B7 are similar in appearance with no blue-halo indicating that division is not inhibited.

mutants display a blue-halo phenotype when grown on minimal glucose medium because of an overproduction of the blue-pigmented antibiotic actinorhodin. This blue-halo phenotype is seen for the strain containing pCMC15, whereas the strain containing pCMC22 (*ftsZ*⁺) has a reddish color, also demonstrated by the strains containing pCMC15-A1 and pCMC15-B7. The colony phenotypes suggested that pCMC15-A1 and pCMC15-B7 were possible intragenic suppressors.

Phase-Contrast Examination of Strains Containing Plasmids with Intragenic Suppressor Mutations

Identifying the restoration of division through the colony phenotypes on a plate is a quick and easy visual screen; however it is extremely important to directly observe the division phenotypes by phase-contrast microscopy.

Coverslip lifts of both possible suppressors were made following growth on minimal medium plates supplemented with glucose and apramycin. Vector alone has no effect on division (not shown). pCMC22 (*ftsZ*⁺) was introduced into HU133 (Δ *ftsZ*) and used as a positive control. As expected, sporulation was seen with HU133 containing pCMC22 (*ftsZ*⁺) and undivided long, thin aerial filaments were present in the HU133 strain containing pCMC15[*ftsZ*(A275V)]. If there was complete suppression, the strains containing pCMC15-A1 and pCMC15-B7 would be identical to the strain containing pCMC22. However an intermediate phenotype was seen when viewing under phase-

contrast microscopy. Both strains containing pCMC15-A1 and pCMC15-B7 were not null for division yet they were not completely sporulating.

The phenotypes observed for the strain containing pCMC15-A1 were slightly coiled aerial filaments and chains of extremely misshapen spores (Figure 8). Coiled filaments were more common than filaments containing the misshapen spore chains.

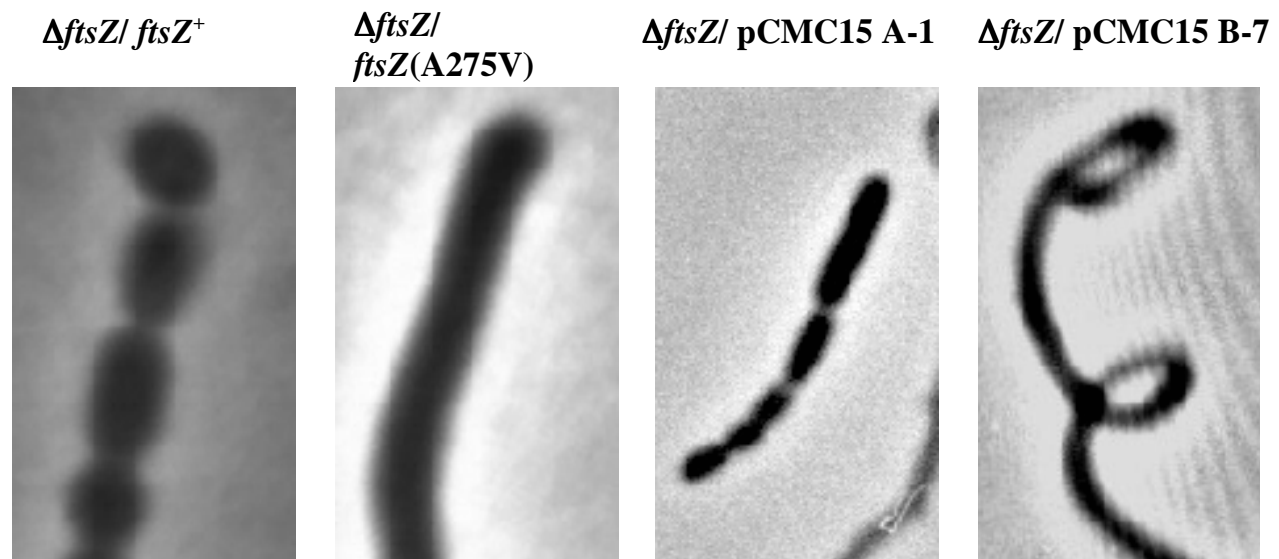


Figure 8. Phase-Contrast Microscopy Indicates that Possible Suppressors of *ftsZ*(A275V) Partially Restores Division. Shown are phase-contrast micrographs of aerial hyphae filaments taken after five days growth on minimal medium with apramycin (30 μ g/ml). Aerial hyphal filaments of two possible suppressors of *ftsZ*(A275V) are seen in the two micrographs on the right. The suppressor mutation contained on plasmid pCMC15-A1 restores division sufficiently to occasionally have spore-like compartments. The coiled aerial filaments seen in the strain containing pCMC15-B7 indicate a later block in development rather than complete loss of division.

The misshapen spore chain result indicates that the original mutation has been partially suppressed and division partially restored. For every five to six misshapen spore chains there was one coiled filament observed in the strain containing pCMC15-A1.

The fact that the aerial filaments are coiled indicates that division in the base of the aerial filament has occurred and development in the remaining portion has been halted (Ryding *et al.*, 1999). Coiled aerial filaments were the only phenotype seen in the strain containing pCMC15B-7.

Verification that the Suppressor Mutations are Associated with pCMC15-A1 and pCMC15-B7

To make sure the phenotypes seen by strains containing both pCMC15-A1 and pCMC15-B7 were due to a mutation associated with each plasmid, each one had to be checked. This was carried out by isolating chromosomal DNA from each potential suppressor and recovering the plasmid by transforming the DNA into an *E. coli* strain. The recovered plasmid DNA was then reintroduced into HU133 (\DeltaftsZ) by conjugation to see the phenotype expressed. The phenotypes observed matched the original phenotypes (data not shown), allowing the conclusion to be reached that the phenotype was associated with the introduced plasmid.

Sequencing results show that for the strain containing pCMC15-A1, an additional mutation exists at position 101 of the protein. The mutation (a G to A transition) changes a glycine (GGT) to a serine (AGT). It appears, from the crystal structure (Figure 9) that

the mutation is located in the GTP-binding region. I am in the process of sequencing the *ftsZ* gene contained on pCMC15-B7.

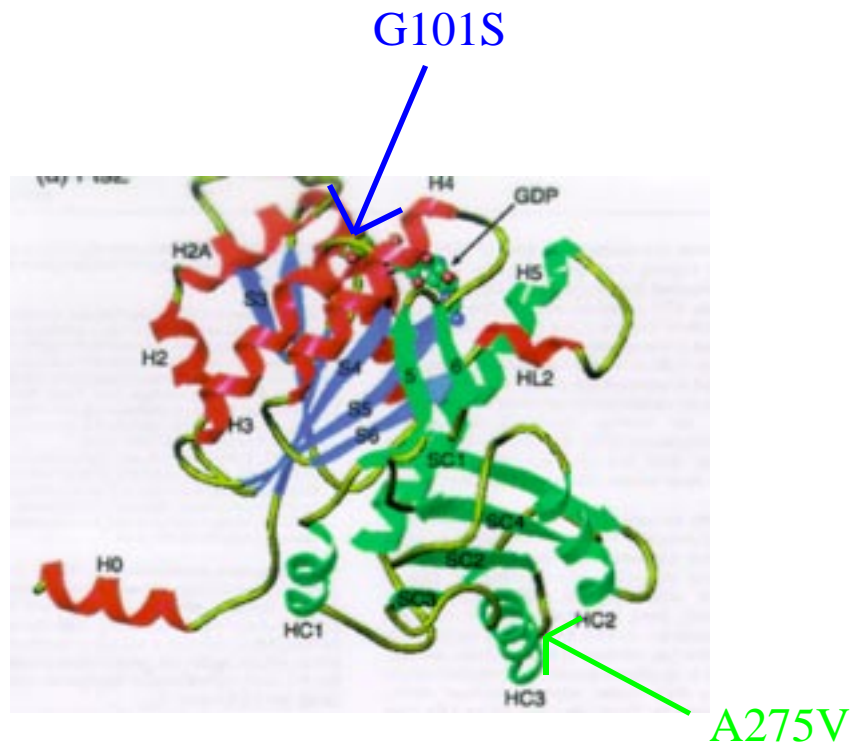


Figure 9. Location of Partial Suppressor Mutation, pCMC15-A1. The location indicated by the blue arrow is G101S. The green arrow indicates the location of A275V. The mutations are located far apart in the monomer but are near one another when subunits assemble into a filament.

Hydroxyl Amine Mutagenesis

One additional way I explored finding potential suppressors was by using hydroxyl amine mutagenesis, a form of chemical mutagenesis. I attempted to use one protocol for hydroxylamine mutagenesis that was successful for isolating another *ftsZ* allele (Grantcharova *et al.*, 2003) but the efficiency of the mutagenesis in my experiments

was not very high and an unexplained loss of DNA was a problem. Included in Table 5 are the results from one of my experimental attempts. Estimating mutagenesis efficiency is by seeing a 99% decrease in transformation efficiency.

Table 5. Estimation of Mutagenesis Efficiency Using Hydroxyl Amine by Measuring the Decrease in Transformation to Antibiotic Resistance

Exposure to treatment (hours)	[pCMC22(<i>ftsZ</i> ⁺)] with NH ₂ OH (colonies)	[pCMC22(<i>ftsZ</i> ⁺)] without NH ₂ OH (colonies)	A257V with NH ₂ OH (colonies)	A275V without NH ₂ OH (colonies)
0	568	996	492	313
10	4	476	302	268
24	12	18	0	88
33	0	0	0	34

However, results observed in several different experiments resulted in a very small decrease in efficiency (data not shown), much lower than the expected 99%. The number of colonies was also very small, roughly only 1500 per set of transformations, and this was with untreated DNA. The desired number of colonies needed for screening should be near 10,000. For unknown technical reasons the experiments were unsuccessful. In 5 attempts, the technique was not reproducible. I never introduced the mutagenized DNA into *S. coelicolor* to screen for mutants.

DISCUSSION

Cell division is a widely studied process in prokaryotes. *Streptomyces coelicolor* is becoming a model organism for the study of cell division. Cell division in this organism is dispensable for growth and viability (McCormick, 1994; McCormick and Losick, 1996). This is extremely important when studying division because null mutations can be isolated for division genes with the cell remaining viable. Moreover, division mutants in *S. coelicolor* overproduce the blue-pigmented antibiotic actinorhodin giving the agar surrounding the colony a blue-halo appearance. This allows for an easy screen for division mutants.

FtsZ is the earliest acting cell division protein and is the bacterial homologue to eukaryotic tubulin (Erickson 1995; Erickson 1998; Romberg, 2001; van den Ent, 2001). The structure of FtsZ is superimposable with tubulin (Erickson, 1998) and is an extremely important protein to prokaryotes, just as tubulin is important to eukaryotes. FtsZ has three identified regions: a large, highly conserved N-terminal region consisting of a six-stranded beta sheet surrounded by two and three helices on each side, required for binding and hydrolyzing GTP (Lowe, 1998; Nogales *et al.*, 1998), a C-terminal domain of unknown function, and a spacer region of variable size and sequence (Liu, 1999; Lowe, 1998; Ma, 1997). The core region of the N-terminal domain is responsible for FtsZ-FtsZ monomer interactions (Wang *et al.*, 1997). The beginning of cell division FtsZ polymerizes to form a ring around the mid-cell known as the Z ring (Addinall and

Lutkenhaus, 1996; Rothfield and Justice, 1997). In addition, FtsZ is a GTPase in which GTP is required for FtsZ-FtsZ interaction (Lu, *et al.*, 2001).

Mutations in *ftsZ* affect cell division in *S. coelicolor* (McCormick *et al.*, 1994; Grantcharova *et al.*, 2003). One such mutation, the main focus of my thesis is *ftsZ25*, referred to here as *ftsZ*(A275V) (J. Morris, Master's Thesis). This mutation prevents division from occurring and also acts as a dominant-negative allele in the presence of a wild-type copy of *ftsZ*⁺ and prevents the wild-type copy from being expressed. The location of the amino acid altered by this mutation is on the HC3 helix (the 3rd alpha helix in the C-terminal domain) near the “bottom” interface involved in FtsZ monomer-monomer interaction. The reason why this mutation prevents division is not known. Several reasons can be suggested as to why division is being prevented. One reason is that the change from an alanine to a valine introduces a larger amino acid into that region. Since this amino acid is located in an area that potentially interacts with 5 additional amino acids, a steric problem could prevent division from occurring. This change could possibly prevent FtsZ from interacting with another FtsZ molecule and inhibit dimerization, inhibiting Z-ring formation. Another reason is that the helix, HC3 in one monomer, is located close to the GTP-binding domain in the adjacent FtsZ monomer. Another possibility is that the change alters the ability of FtsZ to undergo proper conformational change. The larger amino acid in this specific region might interfere with the ability of FtsZ to change from a straight to a curved filament.

Since there was not a clear reason as to why this mutation in *ftsZ* blocked division, suppressor mutation analysis was a way to attempt to see if division could be restored to

the organism. There were two different ways to try to find a suppressor of this mutation: structure-directed mutagenesis and random mutagenesis.

Structure-directed mutagenesis was the first approach that I tried. Using the crystal structure of *Methanococcus jannaschii* FtsZ and a comparison between the primary sequences of *E. coli* and *S. coelicolor* amino acids located in the region of FtsZ(A275V) (highlighted in Figure 10), a change at codon 259 from a leucine to a valine was decided upon. The reason for changing to this specific amino acid was that the wild-type amino acid in the same position of *E. coli* FtsZ (Table 1) and that hopefully changing to a smaller amino acid might relieve a potential steric problem, allowing FtsZ to function correctly. The smaller amino acid (valine) would allow more room in the hydrophobic pocket where FtsZ(A275V) and FtsZ(L259V) exists and allow division to take place. If it was not a steric problem but a problem with dimerization, the thought was that the change of an additional amino acid would alleviate the problem caused by the mutation and allow dimerization to occur, allowing more room in the pocket. Also, in *E. coli* FtsZ, in this region of the protein four of the six amino acids that interact were identical to *S. coelicolor* (Table 1). The first mutation in *ftsZ* of *S. coelicolor* (alanine to valine) made five of the six amino acids identical. The structure-directed change I theorized would make the remaining amino acid of *S. coelicolor* FtsZ in this region identical to the wild-type version of *E. coli* FtsZ. In *E. coli*, this arrangement is fully functional. The idea was that the same combination of amino acids in *E. coli* would work in *S. coelicolor*, allowing for division.

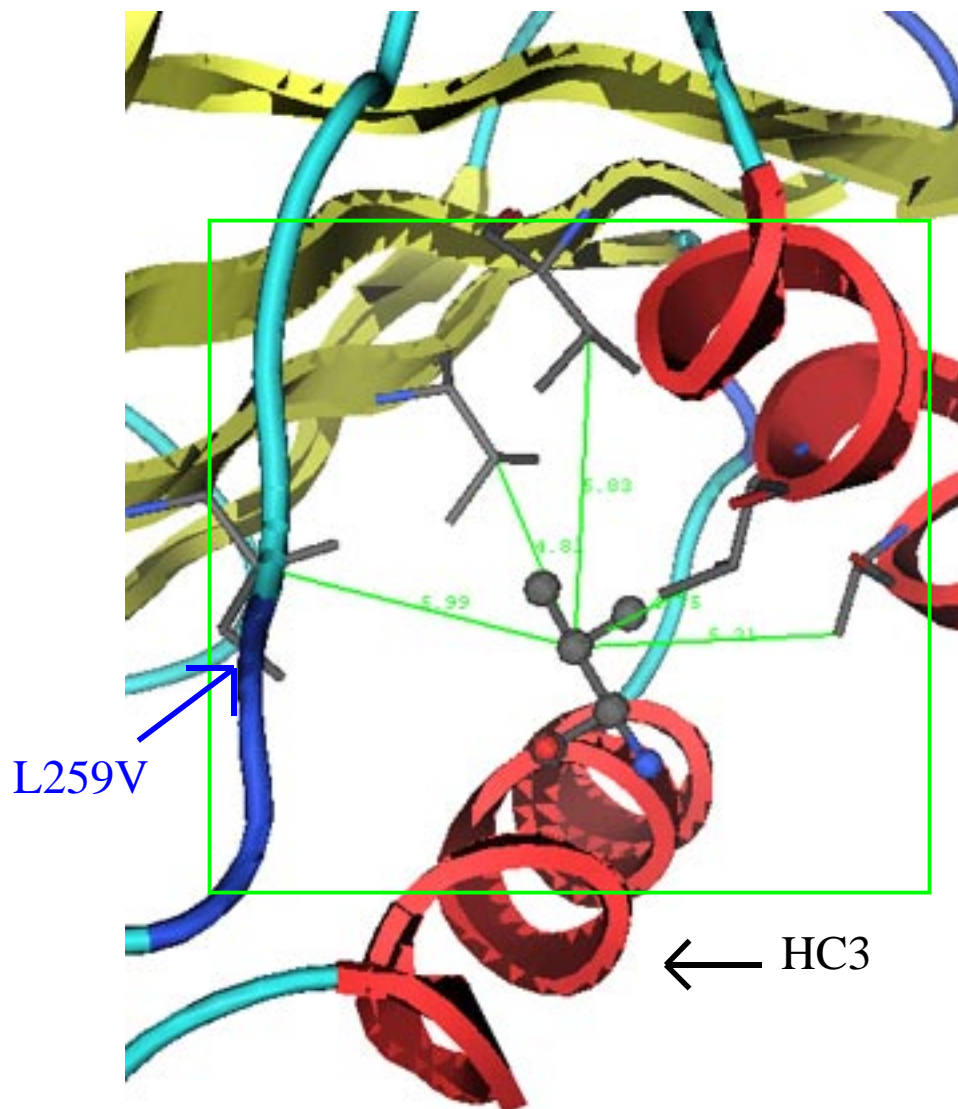


Figure 10. Region of Interaction of 5 Amino Acids in Contact with Valine at Position 275 of FtsZ(A275V). Above is a portion of *Methanococcus jannaschii* FtsZ (Lowe and Amos, 1998). The region boxed in green shows the position of each of the six amino acids located in this hydrophobic pocket. The helix labeled HC3 is the helix that contains FtsZ(A275V). Position 275 is shown in a ball and stick. Positions that interact are shown as stick. The structure-directed amino acid change from leucine to valine at position 259 is also labeled (L259V).

To test the structure-directed hypothesis, two different alleles were created by ligation-mediated PCR. One allele contained a single mutation, *ftsZ*(L259V) and the second allele contained a double mutation, *ftsZ*(L259V, A275V). Both alleles, borne on an integrating vector, were mated into a *ftsZ*-null stain (HU133) and a wild-type strain (M145).

In the strain containing a plasmid expressing *ftsZ* with only the single mutation (pCMC13), division did not occur. This is very interesting in itself because now a conservative change from a larger amino acid to a smaller one in the same region of FtsZ also prevents division from occurring. This region of FtsZ must be crucial for division in the organism if two conservative amino acid changes prevent division. A defect in division was seen in both the *ftsZ*-null stain (Figure 5A, panel 3) and when an additional wild-type copy of *ftsZ*⁺ is present (Figure 5B, panel 3). Just like the previously isolated and characterized *ftsZ*(A275V) (J. Morris, Master's Thesis), my work shows that *ftsZ*(L259V) acts as a dominant-negative over the wild-type. To make sure the phenotype expressed was due to a stable protein accumulating in the cell, a western blot analysis was carried out, comparing wild-type protein to the protein expressed from a gene containing the introduced mutation(s). The western blot (Figure 6B) showed that FtsZ(L259V) accumulates to approximately the same level as when the wild-type protein is expressed in the same context *in vivo*.

In the strain containing the double mutation *ftsZ*(L259V, A275V), the same phenotype was seen as with each single amino acid change. I was expecting that division would be restored. The cell was unable to divide when *ftsZ*(L259V, A275V) was the

only copy present, as in the *ftsZ*-null, (Figure 5A, panel 5) and also was unable to divide when *ftsZ*(L259V, A275V) was expressed in the presence of an additional copy of *ftsZ*⁺ (Figure 5B, panel 5). To test for stability, western blot analysis of FtsZ(L259V, A275V) was carried out. The results indicated that the protein containing both mutations accumulates to levels approximately equal to that of the wild-type and are of the same molecular weight of FtsZ, as seen in Figure 6B. The phenotypes observed are not due to an unstable protein that cannot accumulate *in vivo*. Since the protein is stable, it is reasonable to assume that FtsZ must fold close to the correct confirmation.

The reason *ftsZ*(A275V) cannot divide might not have anything to do with a steric problem in this specific area; however the steric problem could be located elsewhere. Changing to a smaller amino acid (L259V) at another site in the C-terminal domain does not make room for the larger valine and allow proper function. It seems to be that this largely uncharacterized region in the C-terminal domain of FtsZ is extremely important in cell division. Due to the location of both mutations, it is quite possible that FtsZ is unable to function for other reasons such as a GTP-binding problem, FtsZ monomer-monomer interaction, or the FtsZ filament not being able to curve upon GTP hydrolysis.

Further analysis is required for both the single mutation and the double mutation that I introduced into *ftsZ*. Biochemical analysis such as sedimentation assays for polymerization can be carried out to see if there is a polymerization problem occurring. I constructed two plasmids (pCMC17 and pCMC18) to overexpress and purify the altered FtsZ protein to do biochemical analysis. The laboratory will use these in the future. Also since *ftsZ*(L259V) is defective in division, suppressor analysis of this mutation can be

done as well. One additional idea is to explore other amino acids in this region or at least in close proximity of this region and do alanine-scanning mutagenesis, substituting a variety of amino acids with an alanine and see which will result in a dividing organism. Doing something to this effect might give a clearer picture as to what amino acids are critical to a dividing cell.

Since the structure-directed mutagenesis did not yield a suppressor of *ftsZ*(A275V), the second approach was to perform random mutagenesis and use the power of classical genetics.

I tried two different random mutagenesis techniques. The first was a genetic mutagenesis that involved using a mutator strain. The mutator strain being used was XL-1Red, which is deficient in three genes in DNA repair systems. This mutator strain has a 5000-fold increase in spontaneous mutations. Two independent libraries were created and screened for possible intragenic suppressor mutations. Two suppressors have been found (Figures 7 and 8). The two suppressors are in *ftsZ* borne on plasmids, pCMC15-A1 and pCMC15-B7. Neither suppressor completely restores division, as seen in Figure 8. Instead of seeing long, undivided aerial filaments as seen in *ftsZ*(A275V), coiled aerial filaments are seen. The presence of coils suggests a later block in development reminiscent of several *whi* mutants (Ryding *et al.*, 1999). This also implies that division at the base of the aerial filament occurs, allowing part of the division pathway to occur. One suppressor is even more exciting. For the strain containing pCMC15-A1, misshapen spore chains were also seen. Division is restored sufficiently to obtain some spore-like compartments.

Sequencing of pCMC15-A1 suggests that an additional mutation is located upstream of residue 275. The mutation (G to A transition) is located in codon 101 of the gene and changes a glycine to a serine in the protein. This change is located in the GTPase domain in the N-terminus of FtsZ (Lowe and Amos, 1998). The N-terminal domain is considered to be the GTPase domain of FtsZ (Lowe and Amos, 1998). Tubulin also has a GTPase domain and between FtsZ and tubulin the residues involved in this are conserved (Lowe and Amos, 1998). This amino acid change from a glycine to a serine is located two amino acids away from the tubulin signature sequence found in FtsZ, which is as follows: GGGTGTG (Wang *et al.*, 1997). This signature sequence of tubulin is believed to be involved in GTPase activity (Addinall and Holland, 2002). Between FtsZ and tubulin this sequence is chemically conserved and the only difference is a serine in tubulin the sequence, GGGTGSG (Wang *et al.*, 1997). When comparing the sequence of tubulin to FtsZ, at the comparable position in tubulin to the site of the suppressor mutation (G101S), a serine is already present. My hypothesis is that the original mutation, *ftsZ*(A275V), must decrease the activity of the GTP-binding region and *ftsZ*(G101S) must partially restore the activity to this region. Further biochemical analysis is needed to definitively test for the GTPase activity. Thin-layer chromatography can be used to test directly for GTPase activity. Also further analysis can be done on this new mutation. Using site-specific oligonucleotide sequences, this single mutation can be introduced into a wild-type *ftsZ* and the phenotype observed and analyzed. Also, I can also test to see if this mutation will suppress the L259V mutation that I introduced in the structure-directed mutagenesis approach.

Even though at this point in time, two partial suppressors of *ftsZ*(A275V) have been identified, a suppressor, which completely restores division, might still be isolated. Hopefully, in the future, more suppressors will be found and a more definite answer can be found indicating the importance of the HC3 helix in the function of FtsZ.

The second way I attempted to isolate random suppressors was chemical mutagenesis with hydroxylamine. Preliminary experiments with the mutagen yielded no usable results and experimental procedures are still being worked out. Alternative protocols using different amounts of DNA being mutagenized need to be worked out so that enough colonies can be screened. The preliminary experiments I performed yielded only a few hundred colonies, whereas I anticipated that tens of thousands are needed for screening.

Finding more suppressors and discovering the importance of the HC3 helix in the C-terminal domain of FtsZ can also lead to the possibility of finding new cell division genes in *S. coelicolor*. Quite possibly, another cell division protein could be attempting to interact with FtsZ, but cannot interact or signal other proteins because of the A275V mutation. Finding intergenic suppressor mutations could potentially lead to the discovery of other new cellular division proteins in *S. coelicolor*.

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